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CHOLESTEROLEMIA AND CARDIOVASCULAR ABNORMALITIES IN RATS CAUSED BY COPPER DEFICIENCY

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Summary

The association of copper with cardiovascular disease and a possible involvement of copper in the metabolism of cholesterol prompted the study on hypercholesterolemia mediated by copper deficiency. Copper deficient rats were found to exhibit a highly significant cholesterolemia ($P < 0.001$), and plasma cholesterol showed a significant correlation with hepatic copper concentration ($P < 0.03$). Two copper deficient rats died with hemothorax. The hearts of copper deficient rats were hypertrophied with large areas of hemorrhage, inflammation and focal necrosis. Prominent subendocardial fibroplasia was evident in copper deficient animals. The myocardial arteries of copper deficient rats were normal, however, aortas showed large areas of distorted and depleted elastic fibers. The results are discussed in terms of a possible role for copper in cholesterol metabolism, and in the pathogenesis of atherosclerosis.

Key words: *Atherosclerosis — Cardiovascular changes — Cholesterolemia — Dietary copper deficiency*

Introduction

The concentration of cholesterol in human serum [1] and plasma [2] is useful in the prediction of risk of coronary or ischemic heart disease, the

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leading cause of death in the United States [3]. Many environmental factors have been associated with risk of ischemic heart disease; these include high fat [4] and sucrose [5] consumption. Klevay [6—8] has suggested that a relative or absolute deficiency of copper, characterized by a high ratio of zinc to copper [7], is a major factor in the etiology of coronary heart disease. The hypothesis is based upon the production of hypercholesterolemia in rats fed a high ratio of zinc to copper [6] and has been developed in a series of papers [6—13].

Copper has been recognized as an essential dietary component for hematopoiesis since 1928 [14]. Dietary copper deficiency has been shown to produce cardiovascular disease in several species. The first observations of aortic lesions with copper deficient pigs [15] and chickens [16] have been attributed to connective tissue disorders with weakening and rupture of the arteries. Subsequent studies with chicks [17,18] have shown that copper deficiency impairs the cross-linking of lysine residues in collagen and elastin, both of which are dependent upon lysyl oxidase, a copper metalloenzyme [19]. In some cases the weakening of these structures has been sufficient to produce aortic rupture. Myocardial disease without associated vascular lesions has been reported in young rats from copper deficient dams [20]. Myocardial atrophy with an associated fibrosis has been described in cattle with copper deficient diets [21]. Copper is a necessary constituent of mitochondrial cytochrome C oxidase [22], the terminal member of the electron transport chain, and copper deficiency markedly reduces cytochrome C oxidase activity in both liver [23] and heart [20]. Whale and Davies [23] have suggested that the microsomal desaturase activity of rat liver may involve a copper containing protein as the terminal component of the electron transport chain. Such microsomal hydroxylase (monooxygenase) reactions are involved in both the synthesis of cholesterol and the subsequent conversion to bile acids [43—47].

In view of the association of copper metabolism and cardiovascular disease and a possible role for copper in cholesterol metabolism we decided to test the hypothesis that increased cholesterolemia could be produced by copper deficiency.

Materials and Methods

Animals

Weanling male rats (Sprague—Dawley, CF derived strain) weighing approximately 46 g each were obtained from Bio-Lab Corp., White Bear Lake, Minn. *. Rats were assigned to groups of 10 animals matched by mean weight to within 0.4 g. Each member of the experimental group was paired by weight with a member of the control group, and the mean, absolute difference in the weights of the members of each pair was 1.0 g. The rats were housed individually in stainless steel cages and provided with distilled demineralized drinking water ad libitum. Experimental conditions were similar to those described by Klevay

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et al. [24] except extreme filtration of the air was not considered necessary in a non-industrial area.

Experimental diet

All diets were prepared in stainless steel mixing equipment. Table 1 shows the composition of the diet. The rat vitamin mix contained the following components (mg): niacinamide, 4000; calcium pantothenate, 2500; riboflavin, 800; thiamine hydrochloride, 400; pyridoxine hydrochloride, 400; biotin, 200; folic acid, 100; cyanocobalamine, 15; menadione, 10. These components were mixed with sucrose to give a final weight of 500 g. The vitamin ADE mix comprised the following components: calciferol, 5.75 mg; α-tocopherol, 10 g; vitamin A palmitate, 2 g; corn oil to give a total weight of 1000 g.

The basal diet, without the supplementary Zn- and Cu-salt mix, was analyzed by atomic absorption spectrophotometry and found to contain mean copper and zinc concentrations of 0.57 μg and 0.86 μg/g diet, respectively. Animals were maintained on the basal diet for 4 days and then assigned to groups of 10 rats. The experimental group was fed basal diet supplemented to 12.5 μg Zn/g by the addition of reagent grade $Zn(C_2H_3O_2)_2 \cdot 2H_2O$. Similarly the control group were fed basal diet supplemented to 5.0 μg Cu and 12.5 μg Zn/g by the addition of reagent grade $CuSO_4 \cdot 5H_2O$ and $Zn(C_2H_3O_2)_2 \cdot 2H_2O$. The control diet was based on the recommendations of the National Academy of Sciences Committee on Animal Nutrition [25]. In each case the supplementary salts were finely ground and dispersed in powdered dextrose prior to mixing with the diet. Analysis of the supplemented diets showed Zn and Cu to be within 5% of the desired concentrations. On each day each rat in the control group was given an amount of food equal (to the nearest 0.1 g) to that eaten by the member of the pair in the experimental group (pair fed).

Animals were weighed weekly. Blood was collected from the tail vein into heparinized, microhematocrit tubes, and centrifuged. Plasma cholesterol concentration was determined fluorometrically by the method of Carpenter et al. [26]. Liver cholesterol concentrations were determined by a modification of the method of Carpenter et al. [26] following extraction with chloroform—methanol (2 : 1).

At necropsy heart (day 63), aorta and liver were removed promptly from each animal using instruments and containers washed with Radiac metal-

TABLE 1

COMPOSITION OF THE DIET (g/kg)

Sucrose	513.5
Fibrous cellulose powder	30.0
Zn- and Cu-free Jones—Foster salt mix	40.0
Choline chloride	1.5
Egg-white	200.0
Rat vitamin mix	5.0
Vitamin ADE mix (in corn oil)	10.0
Cottonseed oil	95.0
Coconut oil	95.0
Zn- and Cu-salt mix (in dextrose)	10.0

scrubbing soap solution (Atomic Products Corporation, New York, N.Y.), and gloved hands. Blood was obtained by heart puncture and promptly centrifuged. Plasma was removed and placed in metal free tubes. Hearts and aorta were fixed in 10% buffered formalin. Fixed tissues were processed for paraffin sections and cut to a thickness of 6 μm with an American Optical Rotary Microtome, Model 820. Sections were stained with hematoxylin and eosin, or Richardson's trichrome stain.

Metal analysis

Plasma samples were diluted with an equal volume of distilled deionized water and copper and zinc concentrations determined by atomic absorption spectrophotometry (Perkin-Elmer Model 503, Norwalk, Conn.). Diet and liver samples, approximately 2 g each, were weighed in clean, metal free, Erlenmeyer flasks and heated with concentrated nitric and sulfuric acids. Complete digestion was accomplished using 50% hydrogen peroxide. Copper and zinc concentrations were determined by atomic absorption spectrophotometry.

Experimental results were analyzed by Student's *t*-test [27].

Reagents and supplies

Sucrose, Jack Frost, National Sugar Refining Co., Philadelphia, Pa. Fibrous cellulose powder, Whatman CF 11, W. and R. Balston Ltd., London, England. Zn- and Cu-free Jones Foster salt mix, specially prepared by Nutritional Biochemicals Corp., Cleveland, Ohio according to Jones and Foster [28] with the omission of $ZnCl_2$ and $CuSO_4 \cdot 5H_2O$. Egg white, Teklad Mills division of ARS/Sprague—Dawley, Madison, Wis. Choline chloride, Grand Island Biological Co., Grand Island, N.Y. Dextrose, cottonseed oil, coconut oil and vitamins, Nutritional Biochemicals Corp., Cleveland, Ohio. Reagent grade $Zn(C_2H_3O)_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, nitric acid, sulfuric acid, and 50% H_2O_2, Fisher Scientific Co., Pittsburg, Pa. Corn oil, Corn Products Co., Rahway, N.J.

Results

The growth of rats fed a copper deficient diet (0.57 μg Cu, 12.5 μg Zn/g diet) closely matched that of the pair fed control animals (5.0 μg Cu, 12.5 μg Zn/g diet) up to 40 days (Fig. 1). Copper deficient rats ceased growing after 40 days and 2 animals died suddenly with hemothorax on days 49 and 59. Copper deficient rats exhibited mild hypochromic, normocytic anemia. Packed red cell volumes were depressed by 15% at day 45 and 61 (Table 2). Hemoglobin was depressed by 18% at day 63 in copper deficient rats.

By 45 days the mean plasma cholesterol concentration of copper deficient rats was 48% higher than that of pair fed control animals (Table 2). By day 61 this difference had risen to 65% (Table 2). Statistical analysis, using Student's *t* test, showed that at both 45 and 61 days these differences were highly significant ($P < 0.005$ at 45 days and $P < 0.001$ at 61 days). Individual rats fed the copper deficient diet varied considerably in their response to dietary copper deficiency both in terms of packed red cell volumes and increased cholesterolemia. At day 45 the range (69—176 mg/dl) of plasma cholesterol concentrations for copper deficient rats (mean 114 mg/dl, SEM 8.9) overlapped the range

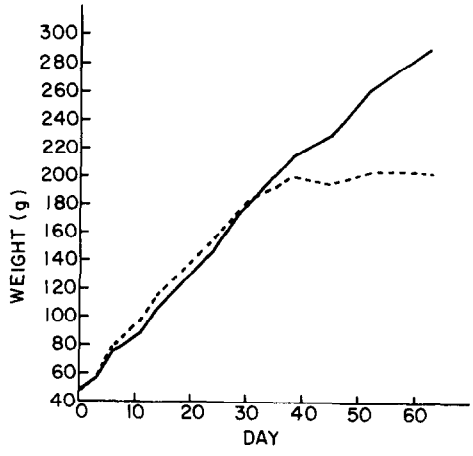

Fig. 1. Growth of copper deficient (- - - - - -) and pair fed control (———) rats.

Fig. 2. Heart of copper deficient rat which died due to heart rupture. Arrow shows the hole in the apex of the heart surrounded by area of necrosis. X 3.5.

TABLE 2

HEMATOCRIT (PCV, %) AND PLASMA CHOLESTEROL CONCENTRATION AT DAYS 45 AND 61 FOR COPPER DEFICIENT RATS AND PAIR FED CONTROLS

	Day 45			Day 61		
	No. of animals	Hematocrit (%, $\bar{x}$ ± SEM)	Plasma cholesterol (mg/dl, $\bar{x}$ ± SEM)	No. of animals	Hematocrit (%, $\bar{x}$ ± SEM)	Plasma cholesterol (mg/dl, $\bar{x}$ ± SEM)
Copper deficient rats	10	43.3 ± 1.3 $P < 0.001$	114.2 ± 8.9 $P < 0.005$	8	42.6 ± 1.6 $P < 0.001$	107.0 ± 4.9 $P < 0.001$
Control rats (pair fed)	10	50.5 ± 1.2	77.1 ± 5.2	10	51.6 ± 1.4	64.8 ± 5.6

TABLE 3

PLASMA AND LIVER COPPER AND ZINC CONCENTRATIONS AND LIVER CHOLESTEROL CONCENTRATIONS OF COPPER DEFICIENT AND PAIR FED CONTROL RATS AT 63 DAYS

	No. of animals	Plasma Cu and Zn (μg/ml, $\bar{x}$ ± SE)		Liver Cu and Zn (μg/g dry liver, $\bar{x}$ ± SE)		Liver cholesterol mg/g wet liver ($\bar{x}$ ± SE)	Mean heart wt (g, $\bar{x}$ ± SE)	Mean heart wt/100 g body wt ($\bar{x}$ ± SE)
		Cu	Zn	Cu	Zn			
Copper deficient rats	8	0.06 ± 0.01 $P < 0.001$	1.10 ± 0.06	2.72 ± 0.30 $P < 0.001$	77.37 ± 4.79 $P < 0.02$	3.71 ± 0.13	1.17 ± 0.15 $P < 0.025$	0.58 ± 0.07 $P < 0.001$
Control rats (pair fed)	10	1.42 ± 0.05	1.05 ± 0.04	12.45 ± 0.78	93.72 ± 3.75	4.02 ± 0.10	0.81 ± 0.04	0.28 ± 0.01

(60—113 μg/dl) of values for the pair fed control animals (mean 77 mg/dl, SEM 5.2). However, by day 61 increased cholesterolemia became apparent in all copper deficient rats and no overlap in plasma cholesterol concentrations occurred between the experimental and control groups.

At 63 days the 8 remaining copper deficient rats and their pair fed controls were killed. No significant differences in liver cholesterol concentrations were observed between copper deficient and pair fed control animals (Table 3). Plasma copper concentrations of rats fed a copper deficient diet showed a 22-fold reduction as compared to pair fed controls ($P < 0.001$) (Table 3). Livers of rats fed a copper deficient diet showed a 78% reduction in copper concentration as compared to pair fed controls ($P < 0.001$) (Table 3). In addition, copper deficient animals showed an 18% reduction in hepatic zinc ($P < 0.025$) despite an identical zinc concentration 12.5 μg/g in both the experimental and control diets (Table 3) and liver zinc concentrations showed a highly significant correla-

Fig. 3. Hematoxylin and eosin stain of the subendocardial region of a copper deficient rat which died due to heart rupture showing active chronic interstitial inflammation with focal necrosis and hemorrhage similar to that seen in myocarditis. X180.

tion with liver copper concentrations ($r = 0.66, P < 0.01$). However, no significant differences in plasma zinc concentrations were found between copper deficient and pair fed control animals (Table 3). These data suggest a role for copper in the maintenance of hepatic zinc stores.

Two out of the 10 copper deficient rats died suddenly with hemothorax and hemopericardium due to heart rupture. Hearts from copper deficient animals were 44% larger than those of the pair fed control animals (Table 3), and this difference was statistically significant ($P < 0.025$), without adjustment for the larger size of control rats at 63 days. One copper deficient animal had a small hole (approximately 1 mm × 1 mm) and necrosis at the apex of the heart (Fig. 2), and death was caused by heart rupture. In several copper deficient rats a large area of hemorrhage was apparent in the subendocardial region. Fig. 3 illustrates a hematoxylin and eosin stain of the subendocardial region from a copper deficient animal which died due to heart rupture and shows hemorrhage and a large inflammatory response with apparent necrosis of muscle fibers. Prominent subendocardial fibroplasia is evident, and the Richardson's trichrome stain (Fig. 4) illustrates the extent of collagen deposition. No aortic ruptures or evidence of elastic tissue abnormalities in intramyocardial arteries was found but regions of depleted and distorted elastic fibers were evident in the aortas of copper deficient rats (Fig. 5) as compared to control animals (Fig. 6).

Fig. 4. Subendocardial section from a copper deficient rat at day 63 stained with Richardson's trichrome stain. Dark areas are collagen and indicate the extent of fibrosis. ×180.

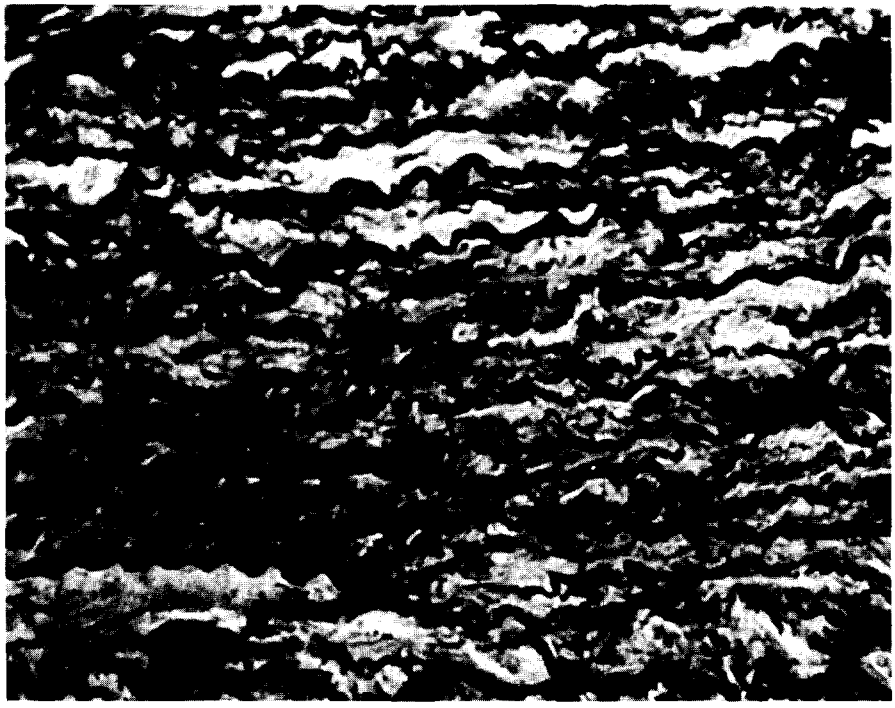

Fig. 5. Aorta of copper deficient rat at day 63 stained with Richardson's trichrome stain showing marked depletion and distortion of elastic tissue. ×320.

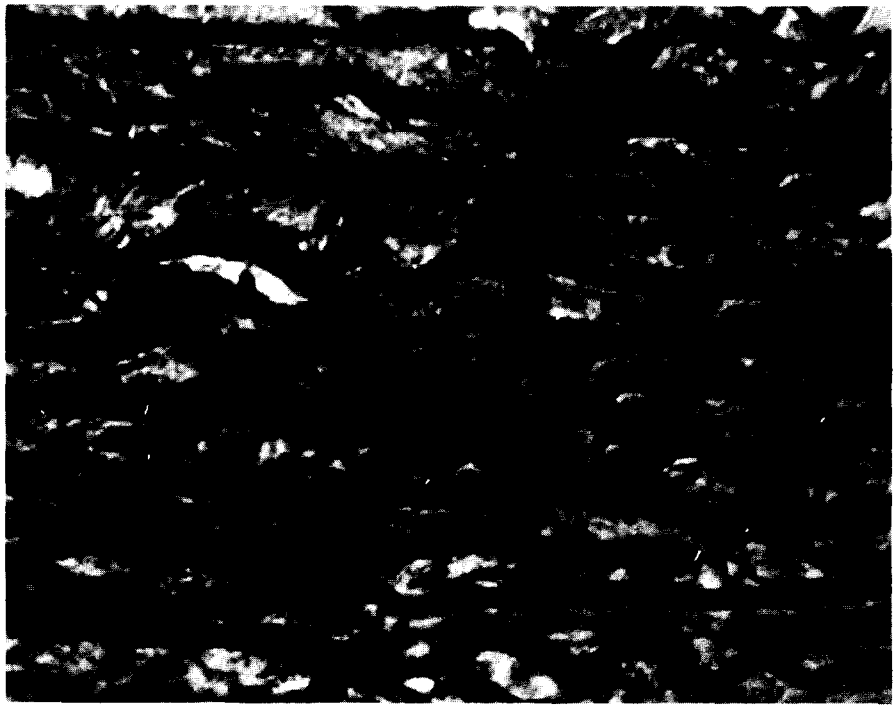

Fig. 6. Aorta from control rat at day 63 showing normal elastic tissue, Richardson's trichrome stain. ×320.

Discussion

The hearts of copper deficient rats were enlarged and pale. Histological examination revealed necrosis of muscle fibers, hemorrhaging, considerable fibroplasia and a large inflammatory response (Figs. 3 and 4). This inflammatory response was more pronounced in those rats dying with hemothorax. The excessive collagen in the hearts of copper deficient rats probably had an abnormal structure since copper is required for the lysyl oxidase dependent cross-linking and maturation of connective tissues [17,18].

Kelly et al. [20] have produced myocardial lesions in young rats by feeding their dams a copper deficient diet during gestation and by feeding the offspring the same diet for 7 weeks after birth. Kelly et al. reported a diffuse fatty change in the myocardium but found no myocardial fibroplasia or aortic abnormalities. In contrast we found large connective tissue changes, but no fatty change. The difference in response to copper deficiency between the experiments of Kelley et al. and those reported here is perhaps due to the intensity and duration of the deficiency. In our experiments lesions were produced in normal rats fed a deficient diet (0.57 μg Cu/g diet) for 49—60 days. The rats of Kelly et al. were fed a diet containing 0.9 μg Cu/g diet and began to experience deficiency in utero. Davis [21] has noted that cattle on a copper deficient diet often show marked atrophy of the myocardium with an associated fibrosis. The collagen deposition in the myocardium results in a weakening of the heart and frequently in death ("falling disease") due to cardiac failure in adult cattle.

The increased cholesterolemia produced by dietary copper deficiency suggests a role for copper in the metabolism of cholesterol. In diets where copper is deficient, or of marginal status, dietary zinc concentration would be of particular significance since a marked zinc—copper antagonism has been observed with marginal copper intakes [29], and a competitive role for zinc in copper nutriture is well documented [30—32]. Rats which exhibited increased cholesterolemia earlier were probably those animals with lower hepatic stores, and the significant correlation of plasma cholesterol and liver copper concentrations ($r = -0.69$, $P < 0.01$) is consonant with this observation. With increasing duration of copper deficiency all experimental animals exhibited increased cholesterolemia as hepatic copper became depleted.

The statistically significant decrease in the liver zinc concentration of copper deficient rats, and the significant correlation of hepatic zinc and copper concentrations, despite an identical dietary concentration of zinc in both the experimental and control groups, suggests a possible role for copper in the maintenance of hepatic zinc stores. This finding is in contrast to the lack of effect of copper deficiency on liver zinc concentrations reported by Alfaro and Heaton [33].

The increased cholesterolemia observed in copper deficiency is attributable to an increase in the synthesis of cholesterol, a decreased synthesis and excretion of bile acids and biliary cholesterol, or to a clearance of cholesterol from the liver to the plasma pool. Despite significant differences in plasma cholesterol concentrations between copper deficient and pair fed control animals (Figs. 2 and 3), no differences were observed in liver cholesterol concentrations (Table

3), suggesting that copper deficiency influences either bile acid synthesis and excretion, or the clearance of cholesterol from the liver to the plasma.

It has been reported [34,35] that fecal elimination of bile acids correlates negatively with serum cholesterol in patients with familial hypercholesterolemia. Similarly, cholic acid turnover is lower in patients with non-familial hypercholesterolemia [36—38]. Since the synthesis and fecal elimination of bile acids is the only quantitatively significant route for cholesterol excretion [39,40] it has been suggested that the synthesis and removal of bile acids, and not the production of cholesterol, are the preponderant factors in determining serum or plasma cholesterol concentrations [41].

The conversion of cholesterol to the primary bile acids, cholic and chenodeoxycholic acids, in the mammalian liver involves 3 hydroxylation steps which are effected by mixed function oxygenases (monooxygenases) [42—45]. Copper has been implicated in several mixed function oxygenases [23,46,47], including the hepatic microsomal desaturation of fatty acids which shows similarities to the 12α-hydroxylation of the steroid nucleus in bile acid synthesis [45]. Since it has been suggested that bile acid synthesis and removal determine plasma, or serum, cholesterol concentrations (vide supra) the increased cholesterolemia caused by copper deficiency is perhaps attributable to a role for copper in the microsomal hydroxylase (monooxygenase) reaction of bile acid synthesis. The major route for the excretion of copper in mammals is via the bile and feces, and it is significant that biliary copper is found in association with the tauro conjugate of chenodeoxycholic acid [48].

The possibility that the increased cholesterolemia caused by copper deficiency is due to a concomitant cellular iron deficiency was considered. However, this explanation is unlikely since dietary iron deficiency does not cause increased cholesterolemia in rats [49,50].

The association of atherosclerotic coronary heart disease with plasma, or serum, cholesterol concentrations is well established. More recent evidence points to an initial injury in the pathogenesis of atherosclerosis [51]. The initial injury is considered to be a focal disruption of the normal vascular endothelial barrier with a subsequent intimal proliferation of smooth muscle cells and an accumulation of connective tissue components and lipids, principally cholesterol and cholesteryl esters, which is often associated with occlusive disease [51]. Recently it has been suggested that the copper metalloenzyme lysyl oxidase, which mediates the cross-linking and structural integrity of vascular connective tissues, may be important in the initial lesion of atherosclerosis [52,53]. Since copper nutriture plays a role in the lysyl oxidase mediated structural integrity of vascular tissue, and also influences plasma cholesterol concentrations, this single dietary component may be involved in both the initial and subsequent steps of atherosclerosis.

Increased cholesterolemia and cardiovascular lesions in animals resulting from copper deficiency may be germane to the problem of atherosclerosis in man. Recent analyses of both institutional [8,11] and self-chosen diets [54] have shown many to contain less than 2 mg Cu/day, the purported daily requirement of average men [55—57]. Since copper plays a well established role in the structural integrity of vascular tissue [16—20,52,53] and copper deficiency also causes increased cholesterolemia, it is possible that the

atherosclerotic process may be mediated to a significant degree by this single dietary component. Copper deficiency may be of importance in the etiology of ischemic heart disease [7], and perhaps provides a link between the initial and subsequent steps in the pathogenesis of atherosclerosis.

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